Supplementary Material For:

A new method for detection and quantification of heartbeat parameters in Drosophila, zebrafish, and embryonic mouse hearts

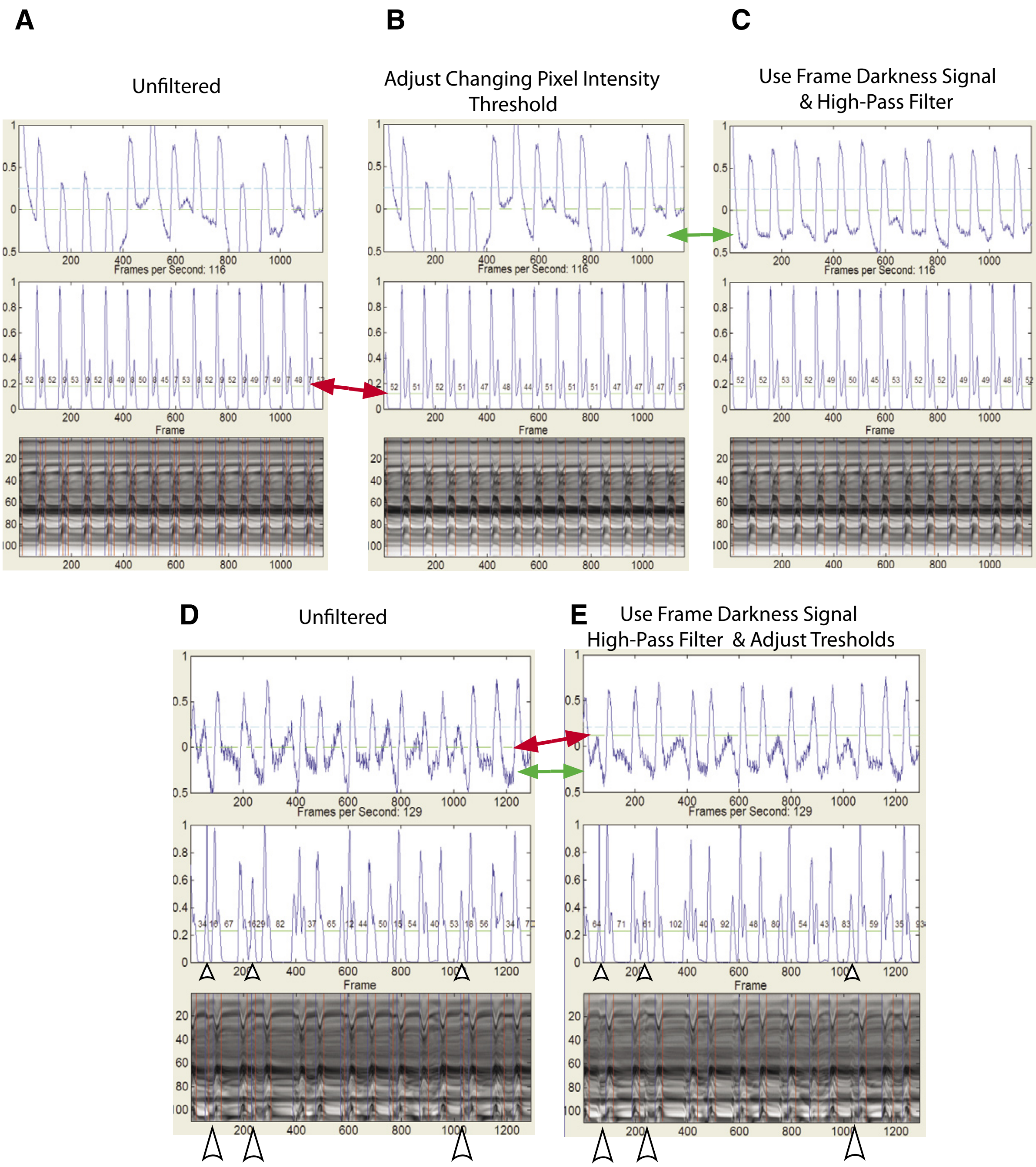
Martin Fink[1,2], Carles Callol-Massot[3,4], Angela Chu[1], Pilar Ruiz-Lozano[5], Juan Carlos Izpisua Belmonte[4,6], Wayne Giles[1,7], Rolf Bodmer[5], and Karen Ocorr[5]

[1]Department of Bioengineering; University of California, San Diego; La Jolla, CA, USA, [2]Cardiac Electrophysiology Group, Department of Physiology, Anatomy and Genetics, Oxford University, Oxford, England, [3]Scientific Department, Biobide, Paseo Mikeletegi 58, San Sebastian, Gipuzkoa, Spain, [4]Gene Expression Laboratory, Salk Institute for Biological Studies, La Jolla, California, USA, [5]Development and Aging Program; Neuroscience, Aging, and Stem Cell Research Center; Burnham Institute for Medical Research, La Jolla, CA, USA, [6]Center of Regenerative Medicine in Barcelona, Barcelona, Spain, and [7]the Faculty of Kinesiology, University of Calgary, Calgary, Alberta, Canada

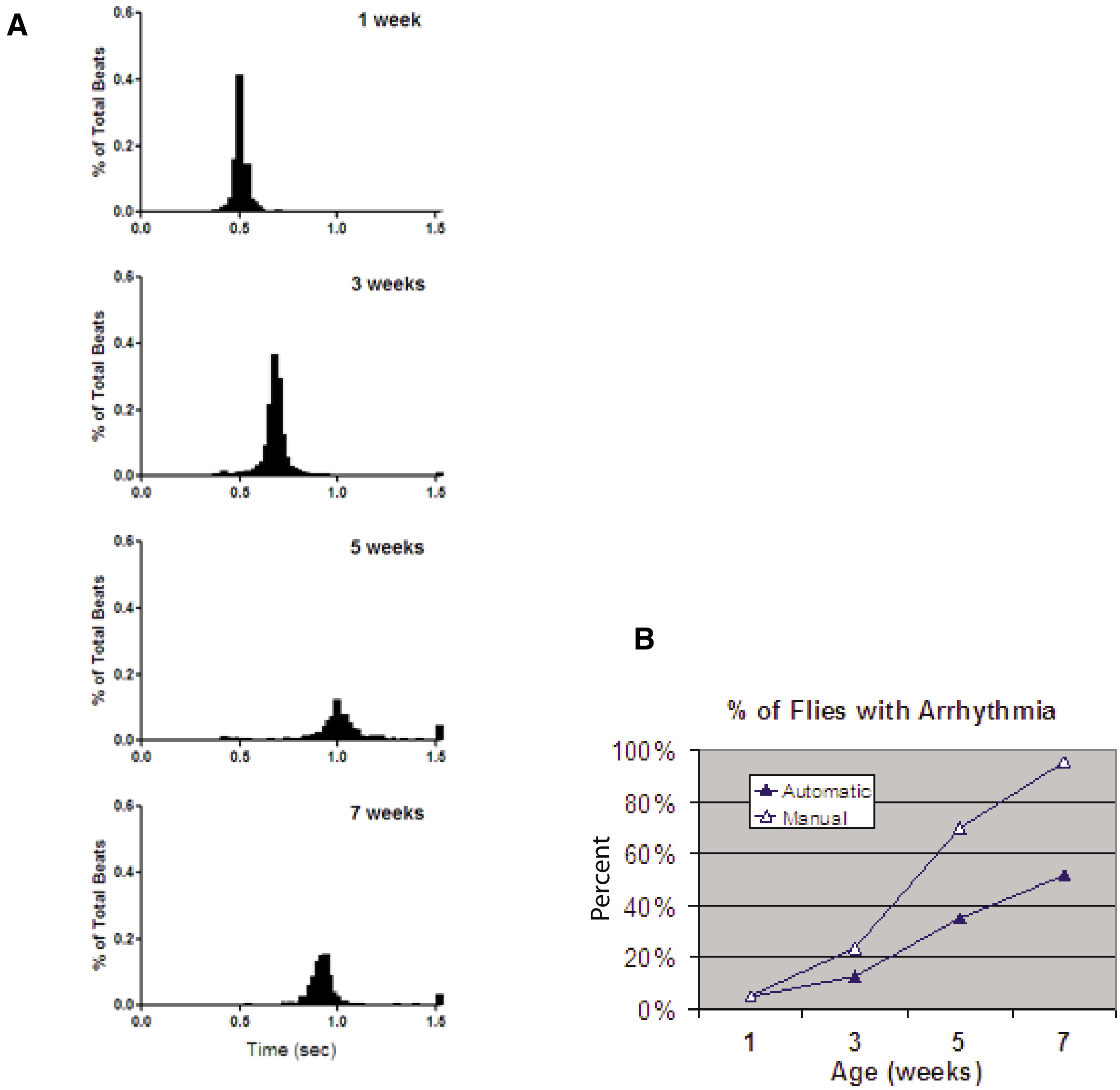
BioTechniques 46:101-113 (February 2009) doi 10.2144/000113078

Supplementary Table 1. Summary of the Statistical Output from Analysis for Young (1-week-old) and Old (7-week-old) Wild-type Flies

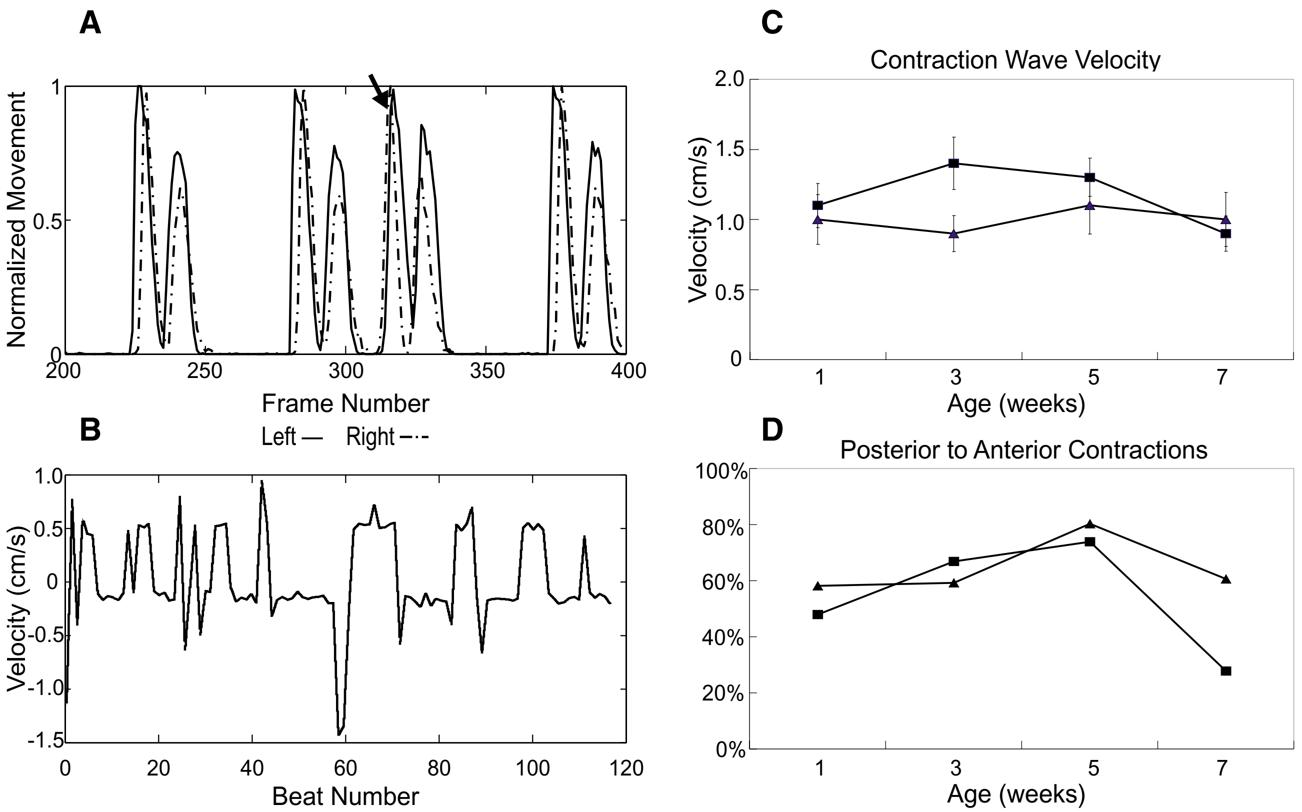
Wild-type		w^{1118}		yw	
Age		1 week	7 weeks	1 week	7 weeks
n		34	24	30	22
Heart rate (Hz)	Mean	2.20	1.56	3.042	1.26
	Median	2.19	1.53	2.49	1.18
	Standard deviation (SD)	0.16	0.32	0.35	0.40
Heart period (s)	Mean	.50	1.01	0.46	1.11
	Median	0.50	0.93	0.45	1.06
	SD (normalized to median heart period)	0.09	0.42	0.17	0.33
Diastolic interval (s)	Mean	0.30	0.60	0.26	0.67
	Median	0.3	0.56	0.25	0.63
	SD	0.14	0.41	0.08	0.32
Systolic interval (s)	Mean	0.20	0.41	0.21	0.45
	Median	0.21	0.38	0.21	0.42
	SD	0.014	0.14	0.02	0.15
Diastolic diameter (microns)		72.3	63.0	80.8	57.7
Systolic diameter (microns)		43.6	39.5	48.4	38.3
Fractional shortening		40	36	40	33
Anterograde contractions (% of total beats, left to right)		49	42	52	71
Anterograde velocity (cm/s)		0.89	1.04	1.00	1.16
Retrograde contractions (% of total beats, right to left)		51	58	48	29
Retrograde velocity (cm/s)		0.99	0.87	1.05	0.92



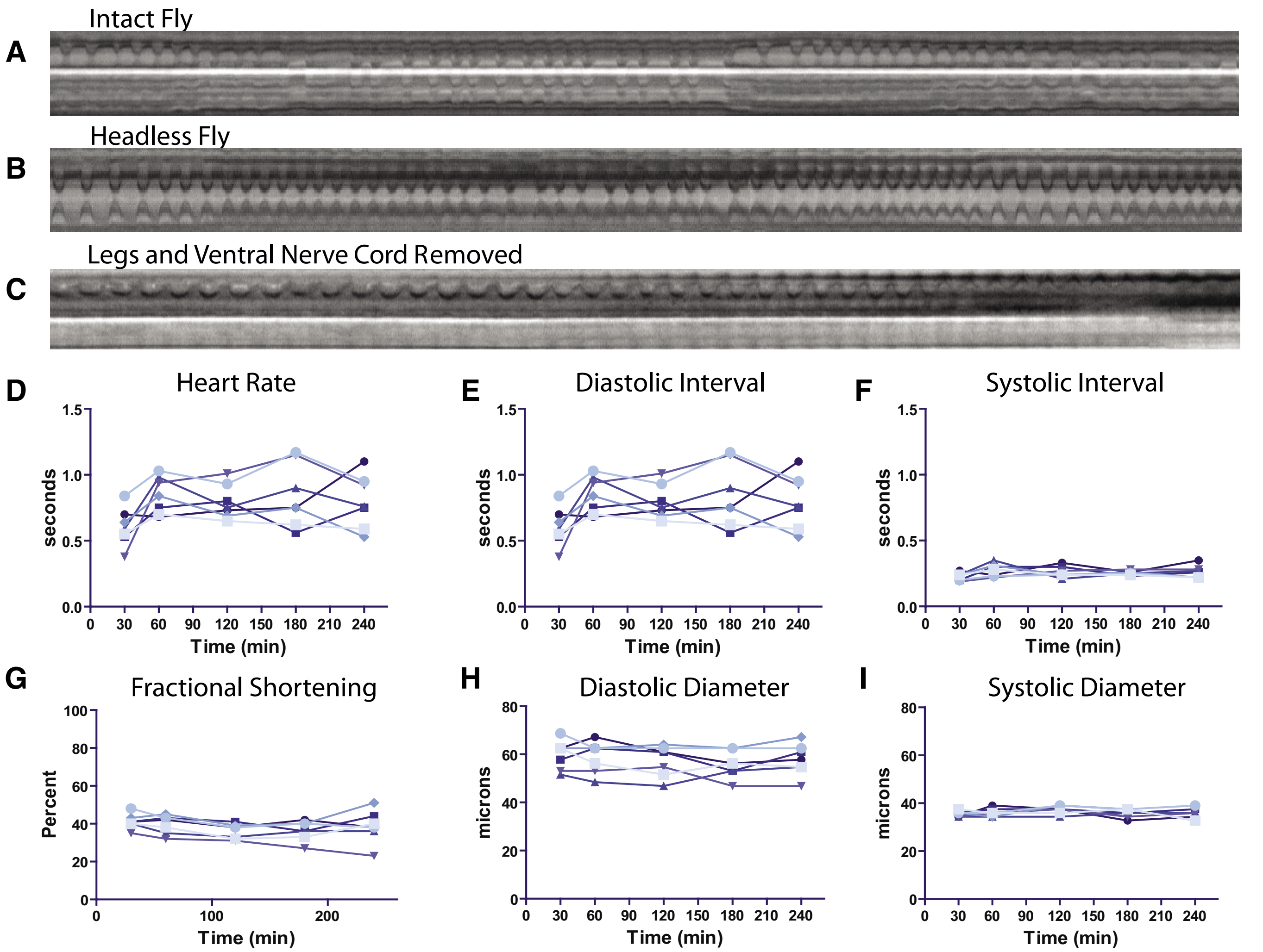
Supplementary Figure 1. Screen shots taken from the Check Intervals module of the program. This interface displays the output from the Frame Darkness (top traces) and Changing Pixel Intensity Algorithms (middle traces) in register with the M-mode showing movement of the heart edges (bottom trace). (A) Unfiltered output from a fly heart movie showing the Frame Darkness signal (top) with a fairly regular occurrence of peaks superimposed on a substantial, low frequency oscillation. The Changing Pixel Intensity signal (middle) also shows a regular occurrence of contraction and relaxation peaks with clear and steep slopes permitting a precise determination of the beginning and end of contractions. The corresponding M-mode (bottom) generated from the same set of movie frames is displayed with the beginning and end of each systolic interval (as indentified by the Changing Pixel Intensity Algorithm), indicated by a blue and red line respectively. Note that in this unfiltered output the brief pause that occurs during a contraction is being detected as a DI for all of the beats. (B) Output from the same movie shown in A after lowering the Changing Pixel Threshold (indicated by the red arrow). SIs are now correctly detected as indicated by the position of the blue and red lines in the M-mode window. (C) Output from the same movie shown in A using the Frame Darkness signal to specify the SIs. The high-pass filter has also been adjusted to filter out the underlying low frequency oscillation seen in A and B (green arrow). SIs are again correctly detected but without the need to adjust the Changing Pixel Threshold as in B. The mean DIs determined using the settings in B or C differ by only 3.7% and the mean SIs differ by only 4.7%. (D) Unfiltered output from a second fly heart movie showing movements of the heart tube that are due to spontaneous contractions of the alary muscles in addition to movements resulting from normal cardiac contractions. Three of these movements are indicated with large arrow heads in the M-mode and their corresponding movement traces in the Changing Pixel Intensity output are identified by small arrow heads. (E) Output from the same movie as in D after adjusting the lower threshold for the Frame Darkness Algorithm (red arrow) and adjusting the High-Pass filter (green arrow). Using the M-mode as a reference SIs are now correctly identified and the mostly one-sided movements associated with the non-cardiac alary muscle contractions are ignored.



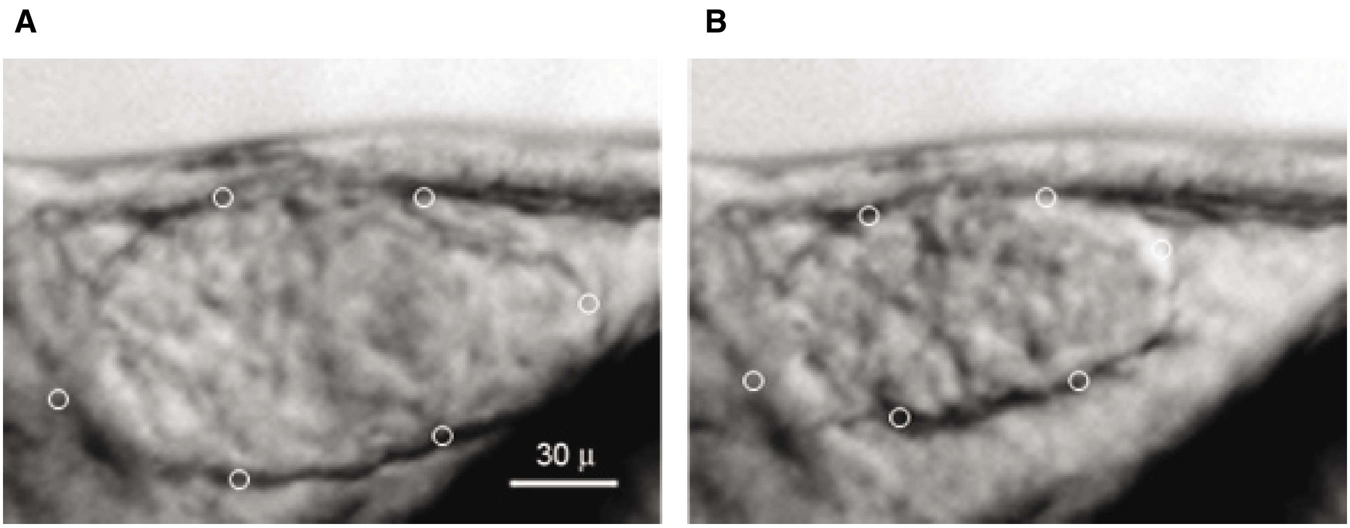
Supplementary Figure 2. Heart period and arrhythmia in flies. (A) Changing distribution of the heart period lengths with age. A single heart period is calculated as the SI length plus the DI length. Data for individual flies were normalized to the median heart period (data for each fly is divided by its median value and multiplied by the group mean); results are presented as the percentage of total beats recorded for all flies in each age group. HP intervals longer than 1.5 s were grouped together and are shown in the last bar of the histogram. (B) Percent of total flies showing arrhythmia. Arrhythmicity in w^{1118} wild-type flies was determined by a visual examination of M-modes from each movie for irregularities (manual) and by the program using the absolute threshold algorithm with DI > 1 s and SI > 0.5 s as cutoffs (automatic). Data are expressed as the percent of total flies for each age group (n = 17–22 flies per data point)



Supplementary Figure 3. Determination of contraction wave velocity. Contraction, and hence movement, occurs in waves that move posterior to anterior (right to left in the movie) or anterior to posterior (left to right). The algorithm for deriving the velocity identifies two locations along the horizontal axis where movement can be optimally detected. Velocity is calculated using the distance between the two regions (converted into a distance in microns based on the movie resolution and microscope calibration) and the time lag between movement signals occurring at each location (time calculated using the movie frame rate). A more detailed description can be found in the Supplementary Algorithm Details. (A) Local movement traces detected at two separate locations (left side and right side) along the horizontal axis in the movie are shown. In the first two contractions, movement peaks on the anterior (left) side of the heart tube before movement is detected on the posterior (right) side. A reversal in pumping direction in the third contraction is indicated by the arrow. (B) Instantaneous contraction velocities during a 60-s movie showing reversals in pumping direction. (C) The velocity of anterograde (posterior to anterior) heart muscle contractions (mean ± SEM, *n* = 17–30 flies per data point). (D) The percentage of total contractions that proceed in a retrograde (posterior to anterior) direction shows an increase with age up to 5 weeks. Squares, yw; triangles, w^{1118}.

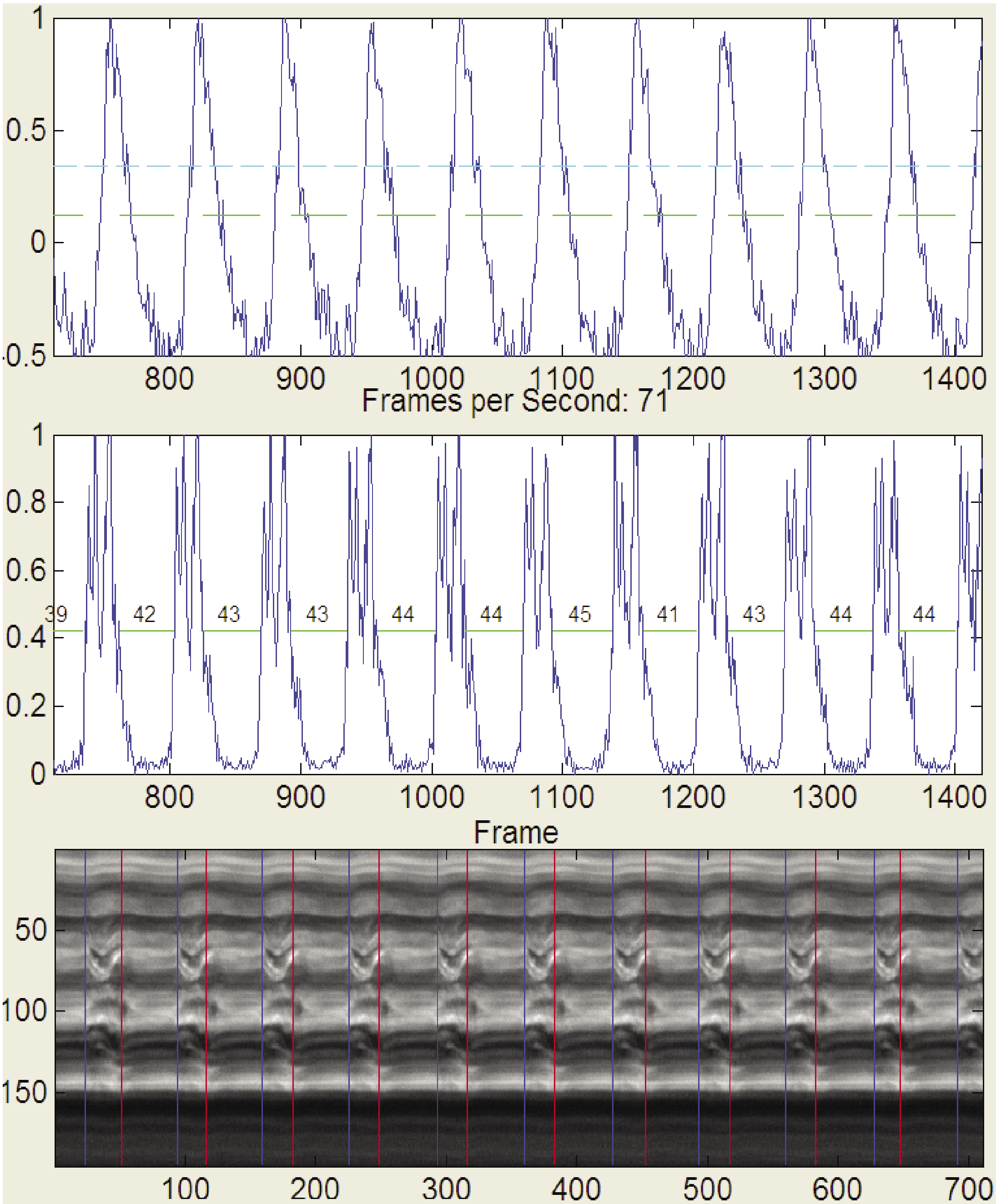


Supplementary Figure 4. Drosophila heart preparation. A–C show the M-mode (10 s) prepared from movies taken through the dorsal cuticle of a single intact wt fly. (A) Immobilized intact adult fly shows a very rapid heart rate (5.0 Hz) interspersed with short period s of more prolonged beats. (B) Removing the head immediately resulted in small increases the rate (5.8 Hz) but did not change the pattern of heart beats (C) Legs and ventral nerve cord were removed and heart contractions were again recorded through the dorsal cuticle. Removing the thoracic ganglia resulted in an immediate reduction in heart rate (to 4.2 Hz) and the establishment of a more regular pattern of heart muscle contraction. (A–C: 4× magnification, dry objective.) D–I show optical recordings from semi-intact Drosophila preparations that were taken following dissection from 30–240 min post-dissection. Seven 4–week-old male w^{1118} flies were used for this analysis. All preparations were maintained in trehalose-supplemented artificial adult hemolymph and were oxygenated in between recording sessions. Data obtained from analysis of the 30-s videos indicates that even relatively elderly fly preparations remain stable with respect to heart rate (D), diastolic interval (E), systolic interval (F), % fractional shortening (G), diastolic diameter (H), and systolic diameter (I) over a 4-h interval following dissection.



Supplementary Figure 5. Measurement of zebrafish heart size. Single frames from a 3-day-old zebrafish movie; anterior is to the left and ventral is up. Frames were identified at the peaks of diastole (A) and systole (B). Two vertical sets of marks and one horizontal set of marks were made. The vertical points are averaged to give the dorsal-ventral width of the ventricle and the horizontal points provide the anterior-posterior length. Surface area was determined based on the equation for the area of an ellipse (see Equation 3), where a = dorsal-ventral width and b = anterior-posterior length.

A
B
C



Supplementary Figure 6. Movement detection in mouse embryos. Mouse embryos (7.5–8 d) were dissected in oxygenated Dulbecco's Modified Eagle's Medium (DMEM) with zero calcium. Isolated embryos were allowed to recover for 1 h and then filmed in situ at 130 fps. Recovery and recordings were performed in oxygenated DMEM containing 1.8 mM calcium at 37°C. (A and B) Output from the movement detection algorithms is shown in the top two windows and the corresponding M-mode is shown in the bottom window. Movement traces look very similar to those seen for the fly and zebrafish hearts. Detected DIs are indicated as a green line (with the number of frames indicated above the line) and SIs are the periods between green lines that correspond to the movement peaks in. (C) M-mode recorded from the same movie frames as in A and B, showing movement of heart edges (y-axis) over time (x-axis). The detected DIs (period from one red line to the next blue line) and SIs (period from one blue line to the next red line) from B agree very well with the pattern of ventricular contraction and relaxation evident in the M-mode.

Supplementary Algorithm Details: Heart Beat Analysis Program

1. Work flow

1. Heart activity is recorded digitally and exported to an AVI movie file.

2. In the "Preprocess" mode, the user manually advances the movie to find frames where the heart is maximally contracted and relaxed and marks the respective systolic and diastolic heart diameters (see in-text Figure 1, A and B).

3. In the "Derive Movement" mode, the algorithms automatically derive the Frame Brightness signal and the Changing Pixel Intensity signal.

4. In "Check Intervals" mode, the user compares the automatic detection of diastolic and systolic intervals (DIs and SIs, respectively) with the M-mode. This interface includes all the information presented in in-text Figure 2, A–C (see also Supplementary Figure 1). The beginning and end of each systole are indicated by lines overlaid on an M-mode image; the user first looks for undetected or extraneous SIs or DIs. Possible issues:

- **Short pauses.** If the Changing Pixel Intensity signal shows only short pauses during the contractions, they will be automatically removed. In this case, the automatic output will match the beginning and end of contraction displayed in the M-mode (as shown in in-text Figure 2, A–C), and no adjustments are needed by the user.

- **Long pauses.** The Changing Pixel Intensity Signal shows longer pauses (i.e., long SIs) or other irregularities that lead to incorrect discrimination of diastole and systole. The user can then turn on the use of the Frame Brightness Algorithm (a check box), in which case output from this algorithm will also be employed to distinguish between systolic and diastolic intervals (compare in-text Figure 3B with Supplementary Figure 1, A and C). Adjustments in the Changing Pixel Intensity Threshold can also be used to correctly distinguish diastole (see below).

5. If the computer-identified start and end of contractions still do not agree with what is displayed in the M-mode, this can be fine-tuned by adjusting a high-pass filter and three thresholds:

- **Threshold for the Changing Pixel Intensity signal.** This "Move" (movement) threshold can be slightly adjusted from the default value in order to have the algorithm ignore the pause in movement that occurs during systole (compare Supplementary Figure 1, A with B) or to ignore noise due to movements that are not part of an overall contraction as reflected in the M-mode. Small adjustments of this threshold setting result in only minimal changes in the output (see Supplementary Figure 1 legend).

- **High-pass filter.** If the Frame Brightness signal contains low frequency oscillations resulting from fluctuations in background illumination these can be removed by using information from the Frame Darkness signal and application of the high-pass filter (indicted by green arrows in Supplementary Figure 1).

- **Two thresholds for the Frame Brightness signal.** These thresholds can be adjusted to ensure the proper distinction between DIs and SIs without adjusting the Changing Pixel Intensity "Move" threshold (compare Supplementary Figure 1, A with C). Peaks within the "window" defined by these two thresholds are used to identify an interval as systolic (i.e., distinction is Boolean). In addition, these thresholds can sometimes be positioned above peaks caused by movements that are not due to heart contractions (compare Supplementary Figure 1, D with E). In this case the program will ignore a portion of the Changing Pixel Intensity signal.

6. If, despite the fine tuning described above, there are still undetected SIs or DIs (with and without use of the Frame Brightness Algorithm) or the detected intervals do not agree with the contractions displayed in the M-mode the data set is discarded for the automated measurements. However, these movies can still be used to generate M-modes for visual inspection.

7. In the "Output Statistics" mode, the software automatically derives DI and SI values for all the contractions in the movie and provides these values along with the other statistics described in the test (including the automatic Contraction Wave Velocity) in a comma-separated value (CSV) file.

Additional output

1. In the "M-mode" module the user can specify specific regions for the derivation of the contraction wave velocity.

2. Additionally, in the "M-mode" module" the user can obtain M-modes from specified locations in the movie frame. M-modes can also be generated with an overlay of the Changing Pixel Intensity trace.

3. In the "Movement Movie" module the user can obtain a reduced speed (1:4) version of the original movie where the pixels detected as moving by the algorithm are colored in red.

2. Mathematical concepts for the developed algorithms

2.1. Nomenclature

Frame. The matrix containing the still image of a frame of the movie
Pixel intensity. The intensity/brightness value of a pixel in grayscale, ranging [0 … 255].
fps: Frames per second in a movie.
The pieces of code given in this supplement follow the notation of Matlab.

2.2 Algorithm for movement detection and interval classification

Frame Brightness Algorithm. The Frame Brightness Algorithm calculates the mean pixel intensity per frame.

```
for i = 1:numberOfFrames
FB(i) = mean(mean(movieFrame{i})); end
```

This signal is normalized to the interval of [0 … 1].

```
FB = FB -min(FB);
FB = FB / max(FB);
```

The Frame Brightness Signal is contaminated by low frequency oscillations due to fluctuations in background illumination, which can be removed using a high-pass filter where the user can specify the filterThreshold.

```
filterLength = fps*3;
filterObject = mov_fir(filterLength, filterThreshold, 'high');
filterSample = conv(filterObject, FB);
FB = filterSample(filtLength/2+1 : end-filtLength/2);
```

The result is always shown to the user (see in-text Figure 2A) and can be used for the interval classification as given below.

Changing Pixel Intensity Algorithm. The Changing Pixel Intensity Algorithm derives the relative change of each pixel between two frames. The chosen formulation is more sensitive to intensity changes of darker pixels (matching the fact that we investigate the movement of dark membranes over light background).

```
for i = 1:numberOfFrames-1
relativeChange{i} = abs(frame{i+1}-frame{i})./(frame{i}+1);
maximumChangeInFrame(i) = max(max(relativeChange{i})); end
```

(The addition of 1 avoids division by zero.)

Experience has shown that the background noise level of the signal is constant. In a part of the movie where there is no movement, the maximumChangeInFrame therefore reflects intensity changes within the background noise, that is, the minimum of the maximumChangeInFrame denotes the backgroundNoiseLevel. Moving pixels have to exceed this level to be considered. We then calculate the numberOfMovingPixels per column (y-coordinate, which is perpendicular to the movement of the contraction-wave) per frame.

```
backgroundNoiseLevel = min(maximumChangeInFrame);
for i = 1:numberOfFrames-1 numberOfMovingPixels{i} = … sum(relativeChange{i} > backgroundNoiseLevel); end
```

This signal is further used for calculating the velocity of the contraction wave as shown later in this supplement. Furthermore the Changing Pixel Intensity Signal, which is used for the interval classification algorithm is the sum of the numberOfMovingPixels per frame.

```
for i = 1:numberOfFrames-1
CPI{i} = sum(numberOfMovingPixels{i}); end
```

It is normalized to the interval of [0 … 1] as presented for the Frame Brightness Signal.

Comparison of the Frame Brightness Algorithm and the Changing Pixel Intensity Algorithm shows that the latter is less noisy, and that peaks indicating contraction and relaxation movements have a much sharper onset and end (correlating with the contractions in the M-mode) and thus is the preferable signal for obtaining the time-points of beginning and end of the contractions.

In-text figures 2C, 2E, and 3B show that the default movementThreshold of 0.2 provides a good estimate of the appearance of movement within the movie, neglecting minor local movement. Note, that the movementThreshold can be adjusted by the user if necessary. Thus the frames in which no movement appears (diastole) can be obtained by

```
frameInDiastole = CPI > movementThreshold;
```

From this one can calculate the beginning and the end of the diastolic interval (DI) and thus heart rate. In some records there are measurable pauses between the contraction and relaxation movements (i.e., a period of no movement) consequently the Changing Pixel Intensity Signal falls below the threshold and these pauses would wrongly be considered to be a DI. In this case the user can employ the Frame Brightness Signal to inform the Interval Classification which intervals belong to the DI and which to the SI, and thus override the classification using the basic movement threshold, even though the borders of the intervals are still determined by the Changing Pixel Intensity Algorithm (compare Supplementary Figure 1, A with C).

We remove DIs (i.e., convert them to be part of the surrounding SIs) whose mean Frame Brightness Signal is above the highBrightnessThreshold and afterwards remove all SIs (i.e., convert them to be part of the surrounding DIs) whose mean Frame Brightness Signal is below the lowBrightnessThreshold. Both thresholds are set by the user to obtain the classification that matches the M-mode of the movie. Finally we remove intervals (i.e., convert them) whose length is shorter than 1/20th of a second.

2.3 Algorithm for obtaining an automatic estimate of the contraction wave velocity

Automatic Detection of Optimal Regions for Determining Contraction Wave Velocity. To increase the accuracy of the velocity measure of the contraction wave (from left to right or vice versa), two regions in the movie have to be selected that lie as far apart as possible; that is, each region contains 15 vertical pixel strips (columns). Due to the differences in the movies and in the illumination of the heart, not all regions provide a strong and robust signal. Therefore, we use the column-wise information that was derived in the Changing Pixel Intensity Algorithm and obtain the meanMovingPixelsPer15Strip for all the 15-strip regions along the x-axis. We assume that the signal should be good enough for our purposes if the normalized meanMovingPixelsPer15Strip is larger than 0.2:

normMovingPixelsPer15Strip = meanMovingPixelsPer15Strip...
/max(meanMovingPixelsPer15Strip);
regionWithGoodSignal = normMovingPixelsPer15Strip > 0.2;

This determines how many regions provide a reasonable signal. As we want to have a signal as strong as possible, but as far apart as possible, we split the regionWithGoodSignal into four and determine the best region in the first and the last quarter, our "optimal" regions (xCooLeft and xCooRight) for determining the Contraction Wave Velocity.

numberOfGoodRegions = sum(regionWithGoodSignal); oneQuarterOfGoodRegions = round(numberOfGoodRegions/4); possibleRegionsLeft = find(regionWithGoodSignal,

oneQuarterOfGoodRegions,'first'); possibleRegionsRight = find(regionWithGoodSignal, oneQuarterOfGoodRegions,'last'); xCooLeft = find(normMovingPixelsPer15Strip == max(normMovingPixelsPer15Strip(possibleRegionsLeft))); xCooRight = find(normMovingPixelsPer15Strip == max(normMovingPixelsPer15Strip(possibleRegionsRight)));

Contraction Wave Velocity Algorithm. This algorithm works similarly to the Interval Classification Algorithm, but we can immediately use the interval classification and the starting frame of each contraction. As the morphology of the movement can be different in the two regions, the starting point of the contraction is the best indicator for the Contraction Wave Velocity. For each contraction and each region, the algorithm determines the first frame where the normalized movement of the 11-pixel strip raises beyond the threshold of 0.2. To obtain a little more accuracy the time-point of reaching the threshold is then interpolated. The Contraction Wave Velocity can then be derived:

CWV=(xCooResolution*xCooDifferenceBetweenRegions)*(fps/timeDifferenceInFrames);

A negative velocity indicates that the Contraction Wave travels from right to left. In the specific case of the Drosophila heart, this provides additional information concerning the dominance of the posterior or anterior.